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Review Article

Natural protease inhibitors to hemorrhagins in
snake venoms and their potential use in
medicine

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Abstract

Snake venoms are complex mixtures of many toxins and enzymes which effectively immobilize prey without a struggle and assist in digestion. Certain animals have a remarkable resistance to envenomation of snakes. Naturally occurring factors that neutralize snake venoms have been found in the sera of most snakes and a few warm-blooded animals. These antihemorrhagic and antineurotoxic factors have been purified from snake and mammalian sera. The antihemorrhagins are not immunoglobulins since they have different physical and chemical characteristics. The natural immunity to hemorrhagins is the result of tissue inhibitors of metalloproteinases (TIMP) found in animal sera of resistant animals. Most animals have matrix metalloproteinases (MMP) and TIMP that are implicated in a wide variety of normal physiological processes and pathological conditions. MMP in animals have many biological functions in embryogenesis, morphogenesis and tissue remodeling. MMP activities are precisely regulated by endogenous TIMP. Disruption of the balance between MMP and TIMP causes various diseases such as arthritis, periodontal diseases, diabetes, ophthalmologic conditions, neoplasia, metabolic bone disease, atherosclerosis and orthopedic conditions. Resistant animals that have a high titer of TIMP would have a survival advantage when bitten by poisonous snakes. Snake venoms are abundant and stable sources of MMP which are medically important. The venom MMP which cause unregulated destruction of tissue have sequences which have some degree of homology with mammalian MMP which control normal biological processes. Resistant animals are important sources of TIMP which can be used to study metalloproteinase related diseases. For these reasons the MMP in snakes and TIMP in resistant animal are excellent candidates for developing new drug therapies. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Snakes are venomous animals that use venom as an effective method of capturing and digesting prey. Snake venom is a complex mixture of many toxins which allows snakes to immobilize their prey without a struggle or chase. When humans are envenomated this creates medical emergencies that must be treated in a timely manner. Most new world poisonous snakes exert an unregulated metalloproteinase attack on cells which cause hemorrhage, edema and necrosis. Matrix metalloproteinases (MMP) are important molecules in normal physiological and pathological processes of most animals and are regulated by tissue inhibitors of metalloproteinases (TIMP). It is the unregulated attack of metalloproteinase on human cells which cause medical emergencies from envenomation of pit vipers. Yet, certain warm-blooded animals and most snakes have a natural resistance to snake venoms and can escape death. The natural resistance found in snakes has been known for almost a century but understanding the mechanisms of venom neutralization in resistant animals has been slow (Noguchi, 1909; Kellaway, 1931; Deoras and Mhasalkar, 1963). It now appears that resistant animals have metalloproteinase inhibitors which endows them with a natural immunity (De Wit and Westrom, 1985; De Wit and Westrom, 1987). Recent interest in MMP and TIMP in cancer and other diseases will greatly increase our understanding of molecular interactions of MMP in snake venom with TIMP in sera of resistant animals.

2. Antihemorrhagins in snake sera

Noguchi (1909) reported that snakes have a natural resistance to their own venom and to venoms of other snakes. No experimental evidence was given as to the mechanism of neutralization but speculated that the lack of receptor sites in snake tissue could be one possible mechanism. Kellaway (1931) reported that Australian venomous snakes possessed a high degree of immunity to their venoms and venoms of closely related species. Experimental evidence was presented to show that the natural resistance was due to the protective properties of the plasma and was thought to be the result of accidental snakebites. Nichol et al. (1933) reported that snakes in the Viperidae family were resistant to their venoms and venoms of other species within the same family. Snakes were encouraged to bite each other; however, the amount of venom injected was not determined.

Swanson (1946) extracted venoms from several species and injected the venoms into the same species as well as other species. The time between death was used as a relative measure of resistance. Snakes appeared to be more resistant to venoms of closely related species. One exception was the copperhead which seemed to be less resistant to its own venoms than to venoms from other snakes. It was concluded that no two venoms were similar in composition and lethal effects.

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Philpot and Deutsch (1956) found that the serum of a kingsnake, *Lampropeltis getulus floridana*, inhibits the proteolytic action of water moccasin, *Agkistrodon piscivorus*, and Eastern diamondback rattlesnake, *Crotalus adamanteus*, venoms. Kingsnake serum inhibited hemoglobin digestion with *C. adamanteus* and *A. piscivorus* venoms but was sixteen times more effective in the inhibition of *C. adamanteus* venom.

Deoras and Mhasalkar (1963) studied the antivenom activity of certain snake sera in mice before and after fractionation. They determined the antivenom activity by injecting mice intraperitoneally with a lethal dose of venom mixed with 0.25 ml of snake serum and by observing the percent mortality. Russell's viper (*Vipera russelli*) serum protected against Russell's viper venom and the rat snake serum protected against the venoms of cobras, vipers and kraits. The genera and species of these snakes were not reported. Russell's viper serum was fractionated with 66% ammonium sulfate. After removing the precipitin fraction, the remaining supernatant possessed antilethal factors which neutralized Russell's viper venom.

Several studies have reported the ability of different species of snakes to neutralize snake venoms (*C. adamanteus*, Clark and Voris, 1969; *C. adamanteus* and *C. atrox*, Straight et al., 1976; *Trimeresurus flavoviridis*, Omori-Sato, 1977; *V. palaestinae*, Ovadia, 1978; and *C. atrox*, Weissenberg et al., 1991). Clark and Voris (1969) reported that the ability of *C. adamanteus* serum to neutralize its own venom in mice was due to serum albumin rather than the immunoglobulin fraction. Whole *C. adamanteus* serum (11.7 mg/kg body weight) heated at 56°C for 30 min, offered excellent protection in mice injected with an LD₅₀ of *C. adamanteus* venom. *C. adamanteus* serum was fractionated on G-200 Sephadex and the antilethal activity was associated with the molecular weight range of 70 to 150 kDa. Analysis by electrophoresis revealed that albumin was the major component of the fraction. Whole Eastern diamondback rattlesnake serum was precipitated with 10% trichloroacetic acid and resolubilized with an ethanol solution. The resolubilized fraction protected at the same degree as the purified Sephadex fraction. All serum proteins are precipitated with trichloroacetic acid but only albumin can be resolubilized with an organic solvent. The results suggest that the antilethal factor was albumin or albumin like molecules. Schaeffer et al. (1978) infused one group of rats with 1 ml of 5% albumin solution over a 0.5 h period which corrected hypoproteinemia, hemoconcentration, lactacidemia and increased the survival rate of rats when injected with Southern pacific rattlesnake (*C. viridis helleri*) venom. All animals given the venom-albumin mixture died. This suggests that the infusion of albumin solution increases the survival time but this is not a result of direct binding of albumin and venom. Straight et al. (1976) reported conflicting results in a later study using the snake serum. They showed through electrophoresis that the antilethal factor in the serum of *C. adamanteus* did not migrate with albumin, but moved at a slower rate.

Omori-Sato et al. (1972) isolated an antihemorrhagic factor from the serum of the habu snake, *T. flavoviridis*. The purified serum factor inhibited two immunologically distinct hemorrhagic principles, HR1 and HR2, in the venom.

The antihemorrhagic factor had a molecular weight of 70 kDa, a sedimentation coefficient of 4.05 and an I_{pH} of 4.0. The purified factor migrated to a position in the area of albumin and α -globulin which was similar to the migration reported by Straight et al. (1976) for the antilethal factor in *C. adamanteus* serum. The antihemorrhagic factor did not form a precipitate with crude venom or with purified hemorrhagic factor in an immunodiffusion test. *C. adamanteus* and *T. flavoviridis* snakes have antivenom serum factors that are not globulins (Omori-Satoh et al., 1972; Straight et al., 1976).

Bonnet and Guttman (1971) found that the serum of the Florida kingsnake, *L. g. floridana* provided protection for mice against *A. piscivorus* venom. This work supported Philpot and Deutsch's Philpot and Deutsch (1956) earlier studies. The ring test, immunodiffusion and immunoelectrophoresis revealed a single precipitant reaction occurring between king snake serum and moccasin venom. This did not mean that other antivenom factors were not present. Ammonium sulfate fractionation of kingsnake serum revealed the presence of antiprotease activity in all fractions which suggest more than one inhibitor in the serum of the kingsnake. With gel electrophoresis, the antiproteolytic fraction migrated with the γ -globulin fractions which led Bonnet and Guttman (1971) to believe that the protease inhibitor was a naturally occurring antibody.

Straight et al. (1976) studied the neutralizing effects of *C. atrox* and *C. adamanteus* plasma on venoms from six species of the family Crotalidae and one species of the family Elapidae. The neutralizing activity of the *C. atrox* and *C. adamanteus* plasma was compared to that of commercial horse antivenom. In most cases the snake plasma proved to be more effective than the antivenom, especially against venoms of the snakes belonging in the Viperidae family. They reported that the venom neutralizing factor in rattlesnake plasma was not albumin or an albumin related compound as indicated by Clark and Voris (1969). The antilethal activity of serum fractionated by polyacrylamide slab electrophoresis was found 1.5 to 2.5 cm behind the albumin band. They agreed that the antilethal factor in *C. atrox* and *C. adamanteus* plasma may be similar to the antihemorrhagic factor found in the habu snake, *T. flavoviridis*, plasma reported by Omori-Satoh et al. (1972).

Ovadia and Kochva, (1977), presented evidence that *V. palaestinae* serum neutralized both the hemorrhagic and the neurotoxic activities of *V. palaestinae* venom. Purification of the antihemorrhagic factor showed that it was a heat stable protein with a molecular weight of 56 kDa and would not form a precipitin line in an immunodiffusion test. These results indicated that the antineurotoxin in the blood of *V. palaestinae* is similar to the antihemorrhagic factor found in the serum of *T. flavoviridis* and of *C. adamanteus*. Clark and Voris (1969) and Omori-Satoh et al. (1972) concluded that the protection observed in all three sera was associated with an albumin-like or α -globulin rather than with an immunoglobulin fraction and would not form a visible precipitate.

Like the antihemorrhagic factor (AHF) from *C. atrox*, the AHF found in the serum of *V. palaestinae* was determined to be similar to that of mammalian origin (Ovadia, 1978). The molecular weight of the AHF was determined to be about 80

it of 70 kDa, a sedimentation factor migrated to a position similar to the migration reported in *C. adamanteus* serum. The test with crude venom or with a test. *C. adamanteus* and *T. flavoviridis* are not globulins (Omori-

of the Florida kingsnake, *L. piscivorus* venom. This work (Sch (1956) earlier studies. The electrophoresis revealed a single band in serum and moccasin venom. These were not present. Ammonium sulfate showed the presence of antiprotease inhibitor in the serum of the venomous fraction migrated with the antigen (1971) to believe that the venom is not a globulin.

The effects of *C. atrox* and *C. adamanteus* on the family Crotalidae and on the activity of the *C. atrox* and *C. adamanteus* commercial horse antivenom. In the test, the antivenom was more effective than the antivenom, in the Viperidae family. They agreed that the antilethal dose of snake plasma was not albumin (Clark and Voris (1969). The crylamide slab electrophoresis showed that the antilethal dose of snake plasma may be similar to the *T. flavoviridis*, plasma reported

that *V. palaestinae* serum showed toxic activities of *V. palaestinae* venom. It showed that it was a heat stable factor and did not form a precipitin line in the test. The antineurotoxin in the venomous fraction found in the serum of *V. palaestinae* (Voris (1969) and Omori-Satoh (1971) served in all three sera was similar to that of mammalian origin. The antilethal dose was determined to be about 80

kDa and the IpH was 4.7. The antihemorrhagin failed to form precipitin lines in immunodiffusion tests which suggested that the AHF of *V. palaestinae* serum was probably an albumin-like α -globulin factor rather than an immunoglobulin (Ovadia, 1978).

Antihemorrhagins found in *C. atrox* were characterized as acidic glycoproteins with a molecular weight ranging from 65 to 80 kDa (Weissenberg et al., 1991). The antihemorrhagins did not form precipitin lines with the venom in an immunodiffusion test. According to the authors (Weissenberg et al., 1991), these results indicated that the antihemorrhagins in *C. atrox* is albumin-like or globulin fractions, very much like those found in naturally resistant mammals.

3. Antihemorrhagins in warm-blooded animals

3.1. Opossum serum

The Virginia opossum, *Didelphis virginiana*, occurs throughout most of the United States and is the only member of the genus whose range spans north of Mexico. The majority of opossums occur in Central America and Northern America (Schmidly, 1983). *D. virginiana* has spread throughout most of North America and is one of the most common undomesticated suburban mammals in the United States (Austad, 1988). Much of the opossums' success stems from its mutualistic relationship with human beings (Austad, 1988), as well as its high adaptability and survival strategies (Stewart, 1993). The Virginia opossum (*D. virginiana*) is one of the most remarkable warm-blooded animals used in biomedical research. The primary use of these mammals is "to study basic biological and behavioral processes" (Cohn, 1989). One of the most intriguing survival strategies of the opossum is their ability to neutralize many different hemorrhagins in snake venoms. Virginia opossums are known to tolerate Crotaline venom without developing hemorrhage, or any other side effects normally associated with rattlesnake venom (Kilmon, 1976 and Werner and Vick, 1977). The LD₅₀ for the Virginia opossum has never been determined because of the amount of venom and number of animals required, but Perez et al. (1979) reported 1,121 mg/kg of *C. atrox* venom caused death in the Virginia opossum with massive hemorrhage.

Kilmon (1976) was the first to report that *D. virginiana* had a natural tolerance to snake venom. Adult Virginia opossums were envenomated by natural snakebites using five different snake species: Eastern diamondback rattlesnake (*C. adamanteus*), timber rattlesnake (*C. horridus horridus*), Eastern cottonmouth (*A. p. piscivorus*), Russell's viper (*V. russelli*) and the common Asiatic cobra (*Naja naja*). All Virginia opossums survived. To better determine the exact amount of venom the opossum could tolerate, Kilmon (1976) reconstituted lyophilized cottonmouth moccasin venom and injected 15 mg/kg. All opossums survived with no side effects. The only noticeable trauma occurred at the site of penetration.

Immediately following intravenous envenomation, there was a drop in arterial blood pressure. Recovery was observed within 10 min. The heart rate increased from 160 to 180 bpm and respirations remained constant. No noticeable edema, ecchymosis nor necrosis were reported.

Werner and Vick, 1977 in a later study reported that Virginia opossums were not able to withstand Elapidae, Viperidae or Hydrophidae venoms. Similar results were found to those of Kilmon (1976) when challenging the Virginia opossum with Crotalinae venoms. *D. virginiana* was able to withstand envenomation by crotaline snakes (Eastern diamondback rattlesnake, *C. adamanteus*; Western diamondback rattlesnake, *C. atrox*; Southern copperhead, *A. c. contortrix*; Eastern cottonmouth, *A. p. piscivorus*; Central American moccasin, *A. bilineatus*, Korean, mamaushi, *A. halys brevicaudus*) at concentrations well above the known lethal dose for susceptible animals (*C. adamanteus*, 1.58 mg/kg; *C. atrox*, 3.03 mg/kg; *A. c. contortrix*, 5.36 mg/kg and *A. piscivorus*, 2.46 mg/kg). The Virginia opossum showed no signs of hemorrhage, swelling nor tissue necrosis. There were some subtle changes in respiration and blood pressure that returned to normal within 5 min.

Unlike the results reported by Kilmon (1976), Werner and Vick (1977) reported that the Virginia opossum succumbed to venoms of the Elapidae, Hydrophidae and Viperidae families. Werner and Vick (1977) found that the Virginia opossum died when subjected to elapid (Indian cobra, Chinese cobra, cape cobra and coral snake), hydrophid (sea snake) and viperid (puff adder) venoms. It assumed that the neutralizing ability was due in part to naturally occurring antibodies; however, this hypothesis was disproved by Menchaca and Perez (1981).

Menchaca and Perez (1981) isolated and characterized an antihemorrhagic factor from serum of *D. virginiana*. According to their study, the AHF had a pH stability from 3–10 and a thermostability from 0–37°C. The AHF was reported to have a molecular weight of 68 kDa and an IpH of 4.1. A ring precipitation failed to show precipitin formation with purified antihemorrhagic factor and crude venom. The antihemorrhagic factor in Virginia opossum was not an antibody but rather some other naturally occurring protein closely associated with albumin. Tarng et al. (1986) also reported isolating antihemorrhagic factors using monoclonal antibodies in affinity chromatography. Polyacrylamide disc electrophoresis revealed two bands, a large band with a molecular weight of 65 kDa and an IpH of 4.8 and a lighter band with a molecular weight of 57 kDa and an IpH of 4.1.

Perales et al. (1989) isolated protecting proteins from the Southern opossum, *D. marsupialis* by a batch DEAE–cellulose procedure that was followed by CM–Sephrose ion exchange chromatography. The proteins were considered to be glycoproteins since the electrophoretic band reacted with periodic acid Schiff staining. SDS electrophoresis showed that the purified proteins were heterogenous and had a molecular weight range of 42 to 58 kDa. The purified fraction effectively blocked the lethal effect of *Bothrops jararaca* venom when jointly injected into laboratory mice. The protective proteins were clearly α -globulins with acidic properties. These findings are consistent with the idea that certain animals

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have a natural immunity to snake venom which is the result of molecules other than antibodies.

A metalloproteinase inhibitor, homologous to human α_1 B-glycoprotein, was isolated from opossum serum by Catanese and Kress (1992). The metalloproteinase inhibitor, termed 'oprin' (opossum proteinase inhibitor), was purified by fractionating opossum serum with ammonium sulphate followed by chromatography on DEAE-Sepharose and Mono Q HR 5/5. Oprin was characterized as having a molecular weight of 52 kDa and an IpH of 3.5. Oprin inhibited most snake venom metalloproteinases, but showed no activity on venom serine proteinases or on bacterial metalloproteinases. *C. atrox* HT-a, the most active hemorrhagic toxin in this venom, was not neutralized by oprin. Reaction products formed an enzyme/inhibitor complex. *C. atrox* α -proteinase (EC 3.4.24.1) was also reacted with oprin (Catanese and Kress, 1992). Complex formation between oprin and *C. atrox* α -proteinase was determined by combining a 2-fold molar excess of oprin with α -proteinase. The reaction mixture was separated on Mono Q HR 5/5. Similar amounts of α -proteinase and oprin were chromatographed separately. The reaction mixture contained a peak which did not correspond to either α -proteinase or oprin. In addition, no α -proteinase peak was detected and the oprin peak was decreased by an amount expected for stoichiometric complex formation. The peaks were pooled and analyzed electrophoretically. Only the complex pool showed two bands in the presence of SDS, corresponding to α -proteinase and oprin. The complex pool showed no proteolytic activity on hide powder azure and had no inhibitory activity against the standard *C. atrox* proteinase mixture.

An opossum liver cDNA library was immunoscreened and clones containing cDNA encoding for part of the open reading frame for oprin were isolated. The cDNA inserts contained nucleotide sequences corresponding to two internal amino acid sequences of oprin which had been separately determined by protein sequence analysis. Protein database screening using a 211 amino acid sequence deduced from one of the cDNA inserts showed no significant homology to known proteinase inhibitors. The α -proteinase and oprin controls from the Mono Q columns showed full activity. Comparisons of amino acid sequence, molecular weight and disulfide content of the two proteins suggest that oprin contains 4 of the 5 domains found in human α_1 B-glycoprotein. Results of this study suggest that oprin may partially account for the resistance of venom in opossums (Catanese and Kress, 1992).

Catanese and Kress (1993) later isolated an α_1 -proteinase inhibitor by fractionating *D. virginiana* serum with ammonium sulphate followed by chromatography on DEAE-Sepharose and phenyl-Sepharose. Traces of serum metalloproteinase inhibitors from *D. virginiana* were removed by affinity chromatography on a protein A-Sepharose antibody column. The proteinase inhibitor was estimated to have a molecular weight of 54 kDa. Opossum α_1 -proteinase inhibitor showed a 51-58% identity with other mammalian α_1 -proteinase inhibitor amino acid sequences. The carbohydrate attachment sites and the reactive site residues (M-S) of opossum α_1 -proteinase inhibitors are identical

to those found in humans (Catanese and Kress, 1993). The opossum α_1 -proteinase inhibitor formed a stable enzyme/inhibitor complex with trypsin, chymotrypsin and human neutrophil elastase but did not react with thrombin or with snake venom serine proteinases. Virtually all the opossum α_1 -proteinase inhibitors retained activity when incubated with crude rattlesnake venoms or purified rattlesnake venom metalloproteinases under which human α_1 -proteinase inhibitor was made inactive. Opossum α_1 -proteinase inhibitors was inactivated by papain which caused limited proteolysis of the active site loop. These results support the hypothesis that certain animals have protein inhibitors that bind to metalloproteinases and neutralize hemorrhagic activity found in snake venom.

The antihemorrhagic properties of the common opossum, *D. marsupialis*, serum have also been studied by Rodríguez-Acosta et al. (1995a,b). Antihemorrhagic fraction was isolated by ammonium sulfate precipitation followed by Diethylaminoethyl (DEAE)-cellulose ion exchange chromatography. Venom from *B. lanceolatus* was neutralized by opossum serum fraction with a molecular weight of 97 kDa. This band was assayed for antivenom activity and found to be more potent than any dose of commercial antivenin (Rodríguez-Acosta et al., 1995a,b). A molecular weight of 97 kDa is considerably higher than reported by others (Huang and Perez, 1980; Perales et al., 1989; Catanese and Kress, 1992, 1993).

Sánchez et al. (1998) reported that the antihemorrhagins (or proteinase inhibitors) in serum of Virginia opossum (*D. virginiana*) neutralizes hemorrhagic activity by binding to hemorrhagins in snake venoms. The binding characteristic of antihemorrhagins in *D. virginiana* serum was used to develop a five-step Western blot. The detection of hemorrhagic proteins were measured indirectly with antihemorrhagins in Virginia opossum serum and with DV-2LD#2, a monoclonal antibody specific for Virginia opossum antihemorrhagins. Snake venoms were separated by native-PAGE, transferred to a Millipore Immobilon®-P membrane and then incubated with crude Virginia opossum serum. The hemorrhagins in snake venom bind to antihemorrhagins in Virginia opossum serum which react with the DV-2LD#2 monoclonal antibody that is specific for Virginia opossum antihemorrhagins. DV-2LD#2 monoclonal antibody inhibits antihemorrhagic activity in Virginia opossum serum when mixed in equal amounts. The inhibition of antihemorrhagins by DV-2LD#2 monoclonal antibody suggests specificity. DV-2LD#2 monoclonal antibody did not recognize antihemorrhagins in gray woodrat (*Neotoma micropus*) serum. The five-step Western blot revealed two well defined bands which represent hemorrhagins found in *C. atrox* venom. Venoms from 15 different snake species were examined to determine the usefulness of the five-step Western blot. Other hemorrhagic venoms (Great Basin rattlesnake (*C. viridis lutosus*), Prairie rattlesnake (*C. v. viridis*), Tancitaran dusky rattlesnake (*C. pusillus*), Northern Mojave rattlesnake (*C. scutulatus scutulatus* type B) and Northern Pacific rattlesnake (*C. v. oreganus*)) had one single band in the five-step Western blot. DV-2LD#2 did not bind to the non-hemorrhagic venoms and reacted with 50% of the hemorrhagic venoms used in this study. The monoclonal antibody, CAH, reacted with all the hemorrhagic venoms except for the venom of the King cobra (*Ophiophagus hannah*) and did

The opossum α_1 -proteinase with trypsin, chymotrypsin, thrombin or with snake α_1 -proteinase inhibitors, snake venoms or purified human α_1 -proteinase inhibitor was inactivated by papain. These results support the inhibitors that bind to found in snake venom.

Opossum, *D. marsupialis*, serum (1995a,b). Antihemorrhagic precipitation followed by immunotography. Venom from reaction with a molecular weight activity and found to be more (Guez-Acosta et al., 1995a,b). Higher than reported by others and Kress, 1992, 1993).

Hemorrhagins (or proteinase inhibitor) neutralizes hemorrhagic activity. The binding characteristic was used to develop a five-step assay where were measured indirectly with DV-2LD#2, a anti-hemorrhagins. Snake venom on a Millipore Immobilon[®]-Virginia opossum serum. The hemorrhagins in Virginia opossum antibody that is specific for monoclonal antibody inhibits serum when mixed in equal 2LD#2 monoclonal antibody. The body did not recognize opossum serum. The five-step present hemorrhagins found in the species were examined to. Other hemorrhagic venoms: rattlesnake (*C. v. viridis*), Mojave rattlesnake (*C. v. oreganus*) had DV#2 did not bind to the non-hemorrhagic venoms used in and with all the hemorrhagic *phiophagus hannah* and did

not react with the non-hemorrhagic venoms. The hemorrhagic binding site of CAH monoclonal antibody and the antihemorrhagin in Virginia opossum are different. The five-step Western blot will be a very useful assay for determining hemorrhagic activity without using live animals.

A comparison of purified antihemorrhagic factors is shown in Table 1. In all cases these antihemorrhagins have characteristics different to antibodies.

3.2. Other warm blooded animals

Natural resistance in several mammalian species have been reported by Phisalix and Bertrand since 1895 (Phisalix and Bertrand, 1895), but only recently have these studies been quantitative. Table 2 list some of the warm-blooded animals that have a natural resistance to snake venoms.

Along with snake and lizard sera, Ovadia and Kochva (1977) studied the ability of six species of mammal sera to neutralize five species of snake venom (*V. palaestinae*, *Echis colorata*, *Pseudocerastes fieldi*, *Aspis cerastes*, *Walterinnesia aegyptia* and *N. nigricollis*). Hedgehog (*Erinaceas europeus*) and hamster (*Mesocricetus auratus*) sera neutralized *V. palaestinae* venom in a mouse protection test. Mongoose (*Herpestes ichneumon*) sera partially neutralized *E. colorata* venom in mice. Mongoose serum protected mice challenged with *E. colorata* and *A. cerastes* venoms and therefore must have humoral factors responsible for neutralization. The mongoose survived the injection of 20 LD₅₀ of *W. aegyptia* and 10 LD₅₀ of *N. nigricollis* venom. The survival of the mongoose to these venoms are not due to humoral factors since mice were not protected when challenged with *E. colorat* and *A. cerastes* venoms.

Perez et al. (1978b) studied 40 species of warm-blooded animals and found that 16 of these animals sera blocked *C. atrox* hemorrhagic activity. Perez et al. (1979) further compared the resistance of three species of warm-blooded animals to *C. atrox* venom. Antihemorrhagic activity was found in all three animal sera (hispid cotton rat, *Sigmodon hispidus*; gray woodrat, *N. micropus*, and Virginia opossum, *D. virginiana*) but very little hemorrhagic activity was found in tissue extract with the highest in gray woodrat (4) and the lowest in the hispid cotton rat (1). The highest antihemorrhagic activity in the three sera was found in Virginia opossum (128) and the gray woodrat and hispid cotton rat was the same (64). The LD₅₀ for the gray woodrat (1,121 mg/kg) was the highest being about 6.5 times higher than the hispid cotton rat (172 mg/kg) and 140 times more resistant than Balb/c mice (8 mg/kg). The LD₅₀ was not determined for the Virginia opossum because of the large amount of venom required; however, 1.38 g of venom was injected i.p. into a Virginia opossum which died within 2 h. This amount of venom was equivalent to the LD₅₀ for the gray woodrat. *In vivo* results indicated that the gray woodrat and the Virginia opossum have very similar resistance to *C. atrox* hemorrhagic activity when injected intracutaneously. Thirty-five mg of venom caused a 50 mm hemorrhagic area in the intracutaneous tissue of Virginia opossum while 35 mg of venom produced a 30 mm hemorrhagic area in woodrats. A smaller amount of

Table 1
Comparison of antihemorrhagins in warm-blooded animal sera

Characteristics of anti-hemorrhagins	<i>S. hispidus</i> ^a	<i>D. virginiana</i> ^b	<i>N. micropus</i> ³	<i>S. mexicanus</i> ^d	<i>E. europaeus</i> ^e	<i>H. edwardsii</i> ^f	<i>D. marsupialis</i> ^g	<i>P. opossum</i> ^h	<i>D. albiventris</i> ⁱ
Heat stability (°C)	0-55	0-57	0-56	0-70	-	0-60	-	-	-
pH stability	3-10	3-10	3-10	2-12	-	2-11	-	-	-
Molecular weight (kDa)	90	68	54	52	780	65	42-58	82.5	43
IpH	5.4	4.1	4.1	4.9	-	-	-	-	-
Electrophoresis	α_1 -albumin	α_1 -albumin	α_1 -albumin	α_1 -albumin	α_2	-	α -globulin	-	-
Proteolytic ^d	no	no	no	no	no	-	no	yes	-
RPT ^k	no	no	no	no	-	no	-	-	-
LD ₅₀ *mg/kg ^l	172	< 1,121 ^o	1,121	53	-	-	-	-	-
LD ₅₀ (mice) ^m	14.2	24.6	29.6	-	-	-	-	-	100 LD ₅₀ of <i>B. jararaca</i>
Protective ratio ⁿ	1.8	3.0	3.7	-	-	-	-	-	-

^aPichyangkul and Perez (1981). ^bMenchaca and Perez (1981). ^cGarcia and Perez (1984). ^dMartinez *et al.* (submitted for publication). ^eDe Wit and Westrom (1985, 1987). ^fTomihara *et al.* (1987). ^gPerales *et al.* (1989). ^hDomont *et al.* (1989). ⁱFarah *et al.* (1996). ^jProteolytic activity as measured on gelatin-coated X-ray film. ^kSera were tested for formation of protein complex in ring precipitation test (RPT). ^lThe LD₅₀ were determined in mice. ^mThe LD₅₀ for *C. atrox* venom in mice protected with 0.5 ml of sera from warm-blooded animals. ⁿThe serum protective ratio is expressed as the amount of protein (mg) of venom required to kill 50% of protected mice divided by the amount of protein (mg) to kill 50% of the unprotected mice. ^oThe LD₅₀ was not determined; however, 1.38 g of *C. atrox* venom was injected i.p. into a Virginia opossum which died within 2 h. This was equivalent to the LD₅₀ for *N. micropus* serum. - = not determined.

ratio^a

^aPichyangkul and Perez (1981). ^bMenchaca and Perez (1981). ^cGarcia and Perez (1981). ^dMarinez et al. (submitted for publication). ^eDe Wit and Westrom (1985, 1987). ^fTomihara et al. (1987). ^gPerales et al. (1989). ^hDomont et al. (1989). ⁱFarah et al. (1996). ^jProteolytic activity as measured on gelatin-coated X-ray film. ^kSera were tested for formation of protein complex in ring precipitation test (RPT). The LD₅₀ were determined in mice. ^mThe LD₅₀ for *C. atrox* venom in mice protected with 0.5 ml of sera from warm-blooded animals. ⁿThe serum protective ratio is expressed as the amount of protein (mg) of venom required to kill 50% of protected mice divided by the amount of protein (mg) to kill 50% of the unprotected mice. ^oThe LD₅₀ was not determined; however, 1.38 g of *C. atrox* venom was injected i.p. into a Virginia opossum which died within 2 h. This was equivalent to the LD₅₀ for *N. micropus* serum. — = not determined.

Table 2
Innate immunity of warm-blooded animals

Common name	Scientific name	Snake venom	Authors
European hedgehogs	<i>E. europaeus</i>	<i>V. berus</i>	Phisalix and Bertrand (1895), (1899), Burton (1969) De Wit and Westrom (1987) Brokow et al. (1997)
		<i>B. asper</i>	De Wit (1982)
		<i>A. c. phaeogaster</i>	De Wit and Westrom (1985)
		nonvenom protease	De Wit and Westrom (1987)
		nonvenom protease	De Wit and Westrom (1987)
		<i>V. palaestinae</i>	Ovadia and Kochva (1977)
Long-eared desert hedgehog	<i>Hemiechinus auritus</i>	<i>B. jararaca</i>	Omori-Satoh et al. (1994, 1998)
		Crotalid	Ognev (1962)
		<i>B. asper</i>	Brokow et al. (1997)
Ichneumon mongoose	<i>Herpestes ichneumon</i>	<i>N. naja</i>	Calmette (1907)
		<i>V. palaestinae</i>	Grzimek (1975) and Ovadia and Kochva (1977)
		<i>B. asper</i>	Brokow et al. (1997)
Indian mongoose	<i>H. edwardsii</i>	<i>N. naja</i>	Deraniyagala (1932) Hinton and Dunn (1967)
		<i>T. flavoviridis</i>	Tomihara et al. (1987)
		28 snake species	Tomihara et al. (1990)
Cape grey mongoose	<i>H. pulverulentus</i>	<i>N. nivea</i>	Jsemonger (1962)
Raccoon	<i>Procyon lotor</i>	<i>C. atrox</i>	Perez et al. (1978b)
Prarie vole	<i>Microtus ochrogaster</i>	<i>A. c. phaeogaster</i>	De Wit (1982)
North American	<i>Taxidea taxus</i>	Crotalid	Billard (1910)
White-eared opossum	<i>D. albiventris</i>	<i>B. jararaca</i> , <i>B. moojeni</i> , <i>B. pirajai</i> <i>B. jararacussu</i>	Farah et al. (1996)
		<i>B. jararaca</i>	Soares et al. (1997)
Gray four-eyed opossum	<i>Philander opossum</i>	<i>B. jararaca</i>	Domont et al. (1989)
Southern opossum	<i>D. marsupialis</i>	<i>B. jararaca</i> , <i>V. russelli</i> <i>B. jararaca</i>	Vellard (1945, 1949)
		<i>C. adamanteus</i> , <i>C. atrox</i> , <i>A. c. contortrix</i> , <i>A. p. piscivorus</i> <i>B. jararaca</i>	Moussatche et al. (1978, 1979, 1980, 1981) and Moussatche and Leonardi (1982) Werner and Faith (1978)
		<i>C. adamanteus</i>	Yates et al. (1979), Perales et al. (1986, 1989, 1992) and Moura-Da-Silva and Tanizaki (1989)
		<i>C. d. terrificus</i>	Neves-Ferreira et al. (1997)
		<i>B. lanceolatus</i>	Perales et al. (1989)
		<i>C. vegrandis</i>	Pifano et al. (1993) and Rodriguez-Acosta et al. (1995a) Rodriguez-Acosta et al. (1995b)

(continued on next page)

Table 2 (continued)

Common name	Scientific name	Snake venom	Authors
Virginia opossum	<i>D. virginiana</i>	<i>C. adamanteus</i> ,	Kilmon (1976)
		<i>C. atrox</i> ,	
		<i>C. h. horridus</i> ,	
		<i>A. c. contortrix</i> ,	
		<i>A. p. piscivorus</i> ,	
		<i>A. bilineatus</i> ,	
		<i>A. h. brevicaudata</i> ,	
		<i>V. russelli</i> ,	
		<i>N. n. kaouthia</i>	
		<i>C. adamanteus</i> ,	Werner and Vick (1977) and Werner and Faith (1978)
		<i>C. atrox</i> ,	
		<i>A. c. contortrix</i> ,	
		<i>A. p. piscivorus</i>	
		<i>A. bilineatus</i> ,	Werner and Vick (1977)
		<i>A. h. brevicaudata</i> ,	
		<i>V. russelli</i> ,	
		<i>N. n. kaouthia</i>	
		<i>C. atrox</i> ,	Perez et al. (1978b)
		<i>A. p. leucostoma</i> ,	
		<i>N. n. kaouthia</i> ,	
		<i>O. hannah</i> ,	
		<i>V. russelli</i> ,	
		<i>B. gabonica</i>	
		<i>C. atrox</i> ,	Huang and Perez (1980)
		<i>C. durissus</i> ,	
		<i>C. horridus</i> ,	
		<i>A. rhodostoma</i> ,	
		<i>A. bilineatus</i> ,	
		<i>V. palaestinae</i> ,	
		<i>V. russelli</i> ,	
		<i>P. fieldi</i> ,	
		<i>T. flavoviridis</i>	
		<i>C. atrox</i>	Menchaca and Perez (1981), Huang and Perez (1982) and Tarng et al. (1986)
		47 different venoms	Soto et al. (1988)
		<i>C. atrox</i> ,	Catanese and Kress (1992)
		<i>C. bascilicus</i> ,	
		<i>B. arietans</i> ,	
		<i>C. adamanteus</i> ,	
		<i>Dendroaspis angusticeps</i>	
		<i>C. atrox</i> ,	Catanese and Kress (1993)
		<i>C. bascilicus</i> ,	
		<i>B. arietans</i> ,	
		<i>C. adamanteus</i> ,	
		<i>V. ammodytes</i> ,	
		<i>Dispholidus typus</i>	

Table 2 (continued)

Authors	Common name	Scientific name	Snake venom	Authors
Kilmon (1976)	Mexican ground squirrel	<i>S. mexicanus</i>	<i>C. atrox</i>	Perez et al. (1978b)
	California ground squirrel	<i>S. beecheyi</i>	<i>C. v. oregonus</i>	Poran et al. (1987)
	Woodrat	<i>N. floridana</i>	<i>A. c. phaeogaster</i>	De Wit (1982)
	Gray woodrat	<i>N. micropus</i>	<i>C. atrox</i> ,	Perez et al. (1978a,b)
			<i>A. p. leucostoma</i> ,	
Werner and Vick (1977) and Werner and Faith (1978)			<i>N. n. kaouthia</i> ,	
			<i>O. hannah</i> ,	
			<i>V. russelli</i> ,	
			<i>B. gabonica</i>	
			<i>C. atrox</i> ,	Huang and Perez (1980)
Werner and Vick (1977)			<i>C. durissus</i> ,	
			<i>C. horridus</i> ,	
			<i>A. rhodostoma</i> ,	
			<i>A. bilineatus</i> ,	
			<i>V. palaestinae</i> ,	
Perez et al. (1978b)			<i>V. russelli</i> ,	
			<i>P. fieldi</i> ,	
			<i>T. flavoviridis</i>	
			47 different venoms	Soto et al. (1988)
			<i>C. atrox</i>	Perez et al. (1978b)
Huang and Perez (1980)	Hispid cotton rat	<i>Sigmodon hispidus</i>	<i>C. atrox</i>	Pichyangkul and Perez (1981)
			<i>C. atrox</i> ,	Huang and Perez (1980)
			<i>C. durissus</i> ,	
			<i>C. horridus</i> ,	
			<i>A. rhodostoma</i> ,	
Menchaca and Perez (1981), Huang and Perez (1982) and Tarng et al. (1986)			<i>A. bilineatus</i> ,	
			<i>V. palaestinae</i> ,	
			<i>V. russelli</i> ,	
			<i>P. fieldi</i> ,	
			<i>T. flavoviridis</i>	
Soto et al. (1988)	Coues rice rat	<i>Oryzomys couesi</i>	<i>C. atrox</i>	Perez et al. (1978b)
	Roof rat	<i>Rattus rattus</i>	<i>C. atrox</i>	
	Mexican spiny pocket mouse	<i>Liomys irroratus</i>	<i>C. atrox</i>	
	Deer mouse	<i>Peromyscus</i>	<i>C. atrox</i>	
	White footed deer mouse	<i>P. leucopus</i>	<i>C. atrox</i>	
Catanese and Kress (1992)	Hispid pocket mouse	<i>Perognathus hispidus</i>	<i>C. atrox</i>	
	Striped skunk	<i>Mephitis mephitis</i>	<i>C. atrox</i>	
	Shrew	<i>Crocidura russula</i>	<i>B. jararaca</i>	Omori-Sato et al. (1998)
	Mole	<i>Talpa europae</i>	<i>B. jararaca</i>	
	Meerkat	<i>Suricata suricatta</i>	<i>N. nivea</i>	Jsemonger (1962)
Catanese and Kress (1993)	Hamster	<i>Mesocricetus auratus</i>	<i>V. palaestinae</i>	Ovadia and Kochva (1977) and Brokow et al. (1997)
			<i>B. asper</i>	Brokow et al. (1997)
			Crotalid	Billard (1909, 1910)
	Dormouse	<i>E. nitela</i>		

venom (1.1 mg) was required to produce a 50 mm hemorrhagic area in the hispid cotton rat. Since there are many hemorrhagins found in *C. atrox* venom, Perez et al. (1979) concluded that there must be more than one antihemorrhagin found in animal sera or that a single antihemorrhagin is capable of neutralizing more than one hemorrhagin.

Huang and Perez (1980) compared the hemorrhagin, hemolytic and proteolytic activities of nine snake venoms. All hemorrhagic venoms were found to be proteolytic using casein and gelatin as the substrate. The Western diamondback rattlesnake was the most hemorrhagic and proteolytic of all venoms tested. Two venoms (*V. russelli* and *P. fieldi*) had very little or no hemorrhagic and proteolytic activity. Three animal sera (hispid cotton rat, *S. hispidus*; gray woodrat, *N. micropus*, and Virginia opossum, *D. virginiana*) readily neutralized hemorrhagic activity in *C. atrox* venom. The only sera that neutralized proteolytic activity was opossum serum which neutralized gelatinase activity in *C. atrox* venom. Crude venom was used in this study which made the results difficult to interpret.

Pichyangkul and Perez (1981) determined that the molecular weight of the antihemorrhagic factor in the hispid cotton rat (*S. hispidus*) was approximately 90 kDa and the IpH at 5.4. Neither the purified factor nor crude serum formed a precipitin line with *C. atrox* venom. The antihemorrhagic factor of *S. hispidus* is probably an α -globulin, which has similar characteristics to antihemorrhagic factors isolated in *V. palaestinae* and *C. atrox* serum.

Huang and Perez (1982) studied the myonecrosis induced by *C. atrox* venom in the gray woodrat (*N. micropus*) at an electron microscopic level. Gray woodrats and control BALB/c mice were injected i.m. with various concentrations of *C. atrox* venom. Gross examination revealed extensive hemorrhage when 250 μ g were injected into mice. Electron microscopic examination showed muscle necrosis in mouse tissue. No extensive hemorrhage or muscle damage was noted until 7.5 and 15 mg of venom were injected i.m. into the gray woodrats. The most prominent damage was swollen mitochondria and destruction of the myofibrils for both mice and woodrats. These results clearly indicate that woodrats are more resistant to the myotoxins in Western diamondback rattlesnake venoms than BALB/c mice.

De Wit (1982) reported that prairie vole (*Microtus ochrogaster*) and woodrat (*N. floridana*) have antihemorrhagins which neutralized hemorrhagic activity in Osage copperhead (*A. c. phaeogaster*). Whole vole serum, when incubated with a minimal hemorrhagic dose (8 μ g), was found to reduce the size of the hemorrhage by 37% and woodrat serum was able to prevent any hemorrhage at a two-fold dilution.

Garcia and Perez (1984) isolated antihemorrhagins from the gray woodrat (*N. micropus*) serum by gel filtration and ion exchange chromatography. The homogeneity of the purified factor was measured by polyacrylamide disc electrophoresis. The factor had an IpH of 4.1, molecular weight of 54 kDa and migrated with α -globulin in electrophoresis. The antihemorrhagins failed to form a precipitate with crude *C. atrox* venom and did not show proteolytic activity with gelatin or hide powder. The antihemorrhagin appears similar to that found in the hispid cotton rat (*S. hispidus*) and in Virginia opossum (*D. virginiana*) sera. The

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from the gray woodrat (*N.*
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 norrhagins failed to form a
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 n (*D. virginiana*) sera. The

studies suggest that the mechanism of neutralization is not enzymatic in nature and the neutralization is not accomplished in a polyvalent binding which would result in precipitation. These studies support the De Wit and Westrom (1985) findings that the antihemorrhagins are protease inhibitors.

De Wit and Westrom (1985) found at least ten protease inhibitors in hedgehog (*E. europaeus*) plasma against the hemorrhagic activity of European viper (*V. berus*) venom. Protease inhibitors in hedgehog plasma were fractionated by gel filtration and the protein fractions were resolved by electrophoresis and tested for trypsin, chymotrypsin and elastase inhibiting activity. The hedgehog protease inhibitors were identified by immunoelectrophoresis and four antiproteases (α_2 -protease, α_1 -protease inhibitor, β -macroglobulins and α_2 -antithrombin) showed homologies with human, rat or swine antisera. In another study, De Wit and Westrom (1987) identified and characterized nine protease inhibitors using plasmin as one more serine protease and two proteases of the other functional classes (metalloproteases and cysteine proteases). Of these nine inhibitors, at least five appear to correspond to previously identified inhibitors while the other four are new, bringing the total number of hedgehog plasma inhibitors to 14.

Three macroglobulins have been characterized as the antihemorrhagic factors responsible for venom resistance in the hedgehog (*E. europaeus*). The authors (De Wit and Westrom, 1987) determined the purity and molecular weight of the protein by polyacrylamide electrophoresis. One major band (termed α -macroglobulin and α - β -macroglobulin: 780 kDa) and three weak bands (670, 550 and 539 kDa, one of which may be the β -macroglobulin) were identified. According to De Wit and Westrom (1987), the macroglobulins identified were all metalloproteinase inhibitors.

Tomihara et al. (1987) isolated three antihemorrhagic factors (AHF-1, AHF-2 and AHF-3) from the serum of the Indian mongoose (*H. edwardsii*). All three AHF neutralized the hemorrhagic activity of the hemorrhagic factors (HR 1 and HR 2) from the venom of *T. flavoviridis*. The AHF had the same molecular weights of 65 kDa. No precipitin lines were formed with the AHF and the venom of *T. flavoviridis* and its hemorrhagic factors, HR-1 and HR-2.

An antihemorrhagic and antiproteolytic factor from serum of the Mexican ground squirrel (*Spermophilus mexicanus*) was isolated using Sephadex G-200 gel filtration, ion exchange DEAE A-50, G-75 gel filtration, and high performance liquid chromatography (HPLC) ion exchange DEAE. The purified factor neutralized proteolytic and hemorrhagic activity of crude *C. atrox* venom. Crude *C. atrox* venom contains seven hemorrhagins and all seven hemorrhagins were neutralized with purified antihemorrhagin in the Mexican ground squirrel serum. The LD₅₀ for *C. atrox* venom in *S. mexicanus* was calculated to be 53 mg/kg body weight which was 6.7 times greater than BALB/c mice (8.0 mg/kg, and 21 times less than *N. micropus* (1,121 mg/kg). The antihemorrhagins had a molecular weight of 52 kDa, IpH of 4.9 and did not react in a ring precipitation test. This suggests that the antihemorrhagin is not an antibody and is reacting in a monovalent manner (in press).

Omori-Satoh et al. (1994) reported that extract from the European hedgehog (*Erinaceus europaeus*) possessed antihemorrhagic activity to hemorrhagins in snake venoms. In a later study, Omori-Satoh et al. (1998) extracted antihemorrhagins from muscle of two additional insectivores (shrew, *Crocidura russula* and mole, *Talpa europaea*). In this same study mice, rats, hamsters and rabbits had negligible amounts.

3.3. The importance of metalloproteinase inhibitors in medicine

To understand the significance of metalloproteinase inhibitors in medicine, it is necessary to review the role of proteinases in normal physiological processes and diseases. Proteinase inhibitors and proteases are in delicate balance with each other to control biological functions. The result is a dynamic system in constant flux which can maintain a steady state.

Proteases act by splitting bonds called peptide bonds that link amino acids together into proteins. Proteases destroy or dramatically alter the function of the substrate (proteins). Proteases were among the first proteins ever isolated and studied. The digestive enzymes, pepsin from the stomach and trypsin from the pancreas, break down the proteins in food into amino acids that can be easily absorbed into the blood for further metabolism. Individual cells also contain proteases. These proteases destroy and eliminate unwanted substances they take in by cells. Scavenger white blood cells, for example, use these enzymes to break down the bacteria, parasites and cell debris they engulf. Sperm cells use proteases to penetrate the outer protein surface of egg cells and achieve fertilization.

Proteases can be classified according to their specificity. Some can split peptide bonds between virtually any pair of amino acids and are considered nonspecific proteases. The stomach protease pepsin is a nonspecific protease, as is the plant protease papain. Others only cleave between certain pairs of amino acids and are considered specific proteases.

The classification scheme most widely used is based on structural similarities within the active sites of proteases. The proteases are named for a particular amino acid that plays a key structural and functional role in the active site. The active sites are the regions on the surface of the enzymes where the actual bond-breaking of other proteins takes place. This is generally a very small region. At present, four types have been described: (a) the serine proteases, (b) cysteine proteases, (c) aspartic acid proteases and (d) metalloproteases (contain an atom of zinc in their active site). The metalloproteinases consist of five subfamilies: thermolysin, astacin, serratin, snake venom and matrixin (Bjarnason and Fox, 1994).

The proteinase like other enzymes makes a snug fit with a short segment of just a few amino acids long with the protein to be cleaved. The protease holds the protein in such a way that a bond between a pair of amino acids within the bound segment can break. As the cleavage occurs, the bound protein with an

m the European hedgehog
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ixin (Bjarnason and Fox,

with a short segment of just
d. The protease holds the
of amino acids within the
he bound protein with an

altered shape leaves the protease surface. The protease then binds to another
intact protein.

Matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases
(TIMP) have been implicated in normal physiological processes such as
embryogenesis, morphogenesis and tissue remodelling. Matrix metalloproteinases
activities are precisely regulated by endogenous tissue inhibitors of
metalloproteinases. The disruption of balance between MMP and TIMP plays an
important role in diseases such as arthritis, periodontal diseases, diabetes,
ophthalmologic conditions, neoplasia, metabolic bone disease, atherosclerosis and
orthopedic conditions. Identification of agents which might inhibit MMP is a
reasonable therapeutic goal.

3.4. Regulator functions of proteases

Perhaps the most critical role of proteases is their regulatory function,
turning on and off the activity of a wide array of hormones and other
signaling molecules. Proteases carry out a number of extremely important
biological processes. The coagulation of blood involves the complex interplay
of a series of protease enzymes and the elimination of these plugs when vessels
have healed is brought about by other proteases. One of the most recent
important advances in the treatment of heart attacks and stroke has been the
development of the so-called clot-dissolving drugs, notably tissue plasminogen
activator (TPA) and streptokinase (Cox et al., 1997; Moreno et al., 1997;
White et al., 1997). Both are proteases that activate yet another protease,
plasminogen, which in turn breaks down fibrin, the protein that forms the
essential portion of blood clots. Snake venoms contain proteinases which
dissolve fibrin clots (Markland and Damus, 1971; Markland et al., 1988, 1994;
Retzios and Markland, 1988, 1992, 1994; Ahmed et al., 1990; Guan et al.,
1991; Baker and Tu, 1996; Tu et al., 1996; Rael et al., 1997). Blood-borne
proteases are also key players in the inflammatory response, the body's first
line of defense against infection.

The signaling of molecules keeps the body operating within safe limits. Many
hormones and other active proteins are intended to have their effect within a
localized area of the body and to persist only for short periods of time. Proteases
play an important part in ensuring that homeostasis occurs. When insulin is
needed to bring down blood glucose levels following a meal, a protease modifies
the structure of insulin to produce active hormone. After insulin has stimulated
the absorption and storage of excess glucose, another protease destroys the
hormone, thereby preventing it from bringing the glucose level too low (Smeekens,
1993; Malide et al., 1995; Steiner et al., 1996).

3.5. *Proteases assist in cell migration*

Cell migration across extracellular matrix (ECM) is required in tissue remodeling and tumor invasion (Chen, 1992; Gailit and Clark, 1994; Sato et al., 1994; Birkedal-Hansen, 1995; Kohn and Liotta, 1995; Basbaum and Werb, 1996; Brooks et al., 1996). Penetration of cells through ECM without massive tissue damage is accomplished by the advancing cell focusing metalloproteinases (Sato et al., 1994; Brooks et al., 1996; Basbaum and Werb, 1996) or protease activators such as urokinase at the leading edge of migration. By focusing metalloproteinases, tumor cells can spread without causing massive damage. Experimental data from studies of human malignancy indicate that these proteinases are induced by tumors in order to reconstruct adjacent normal tissue to allow neovascularisation, tumor growth and metastasis. The precise mechanisms by which proteases alter ECM components is unknown, but Giannelli et al. (1997) reported that matrix metalloprotease MMP2 induces the migration of breast epithelial cell by cleaving laminin-5 (Ln-5), a specific component of ECM.

Matrix metalloproteinases are involved in the invasion and metastasis of human cancers by mediating the degradation of extracellular matrix components. The imbalance between the TIMP and MMP give rise to metastasis. Khokha (1994) reported that the overexpression of TIMP-1 suppresses the metastatic ability of B16-F10 melanoma cells in mice. Sledge et al. (1995) reported that batimastat (BB-94) inhibits human breast cancer regrowth and metastasis in nude mice. Batimastat also binds with a snake venom metalloproteinase (Ht-d) which gives way to the production of potential anti-tumor drugs (Bofos et al., 1996).

3.6. *Proteases are as important to viruses*

Because of their minute size, most viruses carry very few genes and must make maximum use of the minimal genetic information they carry. A single gene is used to make a number of different proteins. The HIV virus has one very long polyprotein that consists of several proteins strung together. The HIV virus uses a protease to cut the long polyprotein into many functional parts before viral assembly can take place. Protease inhibitors are being used with some success as potential drugs to control AIDS (Ermolieff et al., 1997; Hallenberger et al., 1997; Pichova et al., 1997).

Before AIDS, protease inhibitors were designed to inhibit human renin, a protease found in the kidney. This renin plays a key role in the regulation of blood pressure and renin inhibitors are highly effective drugs for controlling hypertension. It has been shown that the HIV proteases inhibitors are structurally and functionally related to renin. Indeed, a number of renin inhibitors that had been developed were quickly shown to work against HIV proteases and work began to build on this foundation to produce molecules that were both highly

l) is required in tissue and Clark, 1994; Sato et al., Basbaum and Werb, 1996; M without massive tissue metalloproteinases (Sato Werb, 1996) or protease migration. By focusing on causing massive damage, many indicate that these adjacent normal tissue metastasis. The precise elements is unknown, but protease MMP2 induces the urokinase-type 1 plasminogen activator (uPA), a specific

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specific in their capacity to inhibit HIV protease and that were chemically suitable for use as drugs. All the HIV protease inhibitors in clinical use today are chemical ancestors of the compounds first developed for renin (Moyle and Gazzard, 1996; Korant and Rizzo, 1997).

3.7. Protease inhibitors

Just as proteases control the activation and destruction of hormones and other biologically important proteins, they are also regulated by another class of molecules known as the protease inhibitors. These substances, many of which are themselves proteins, bind to protease enzymes in such a way as to prevent them from reacting with and cleaving the bonds of hormones and other proteins when they are no longer needed. The result is a dynamic system in constant flux, but all the while maintaining a steady state. Because of the vital regulatory roles, proteases and their inhibitors have become prime candidates for research in drug development.

Four members of the tissue inhibitor of metalloproteinases (TIMP) family have been characterized thus far and are designated as TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The TIMP are capable of inhibiting the activities of all known matrix metalloproteinases and as such play a key role in maintaining the balance between extracellular matrix deposition and degradation in different physiological processes. Accelerated breakdown of ECM occurs in various pathological processes, including inflammation, chronic degenerative diseases and tumor invasion. TIMP-1 and TIMP-2 can inhibit tumor growth, invasion, and metastasis in experimental models which has been associated with their MMP inhibitory activity. TIMP-1 and TIMP-2 are multifunctional proteins with diverse actions. Both inhibitors exhibit growth factor-like activity and can inhibit angiogenesis (Gomez et al., 1997).

Certain protease inhibitors mimic the binding process without cleavage. Structurally, the protease inhibitor has a similar amino acid sequence. Unlike the protein substrate, inhibitor molecules are not split by proteases and remain bound acting as competitive inhibitors by preventing the enzyme from reacting with its normal protein targets. Antihypertensive drugs are also good examples of how protease inhibitors are used in medicine. Protease inhibitors such as captopril and enalapril are among the most potent and widely used antihypertensive drugs on the market. These molecules, called angiotensin-converting enzyme (ACE) inhibitors, which block the action of a protease in the kidneys that normally converts the substance angiotensin into an active form that powerfully constricts blood vessels and raises blood pressure (Coric et al., 1996; Fournie-Zaluski et al., 1996).

Increased MMP activity is detected in a wide range of cancers and seems correlated to their invasive and metastatic potential. MMPs thus seem an attractive target for both diagnostic and therapeutic purposes. Several synthetic matrix metalloproteinase inhibitors (MMPI) are currently being developed.

Preclinical studies are promising as they suggest inhibition of several steps in the metastatic process (Denis and Verweij, 1997). Recently, evidence suggests that MMP may have a more complex role in metastasis and that they may make important contributions at other steps in the metastatic process. MMP are key regulators in the growth of tumors, at both primary and metastatic sites (Chambers and Matrisian, 1997).

3.8. Sources of metalloproteinases and metalloproteinase inhibitors

There are more than 300 species of poisonous snake known in the world and many contain metalloproteinases which cause hemorrhage (Bücherl et al., 1968). Snakes use the proteases in venoms in an unregulated way to capture and help digest prey. Certain animals have a better way of dealing with the unexpected surge of metalloproteinases from envenomation. A snake's unregulated use of metalloproteinases is an effective way of capturing prey. Humans cannot tolerate an unregulated surge of metalloproteinases and envenomation creates medical emergencies which could lead to death. Proteases in venom disrupt the basement membrane of capillaries and cause massive hemorrhage. In most cases venom metalloproteinases are abundant, extremely stable and some are structurally similar to metalloproteinases in mammalian cells. The epididymal apical protein found in the *Rattus norvegicus* (Perry et al., 1992) and the protein PH-30 from guinea pig sperm (Blobel et al., 1992) show intriguing similarities to a variety of snake venom hemorrhagic proteins. The sequence identity between the domains of the mammalian proteases and venom proteases is approximately 20–25%, which is low but does represent continuous homology. Snake venom metalloproteinases will play an important role in developing protease inhibitors for biomedical research. (Blobel et al., 1992; Perry et al., 1992). This gives snakes the distinction of being one of the best sources of stable metalloproteinases for biomedical research.

Certain warm-blooded animals and most snakes have antihemorrhagins (protease inhibitors) in their sera which neutralize hemorrhagic activity in venoms. An important question is: do protease inhibitors (antihemorrhagins) in resistant animals evolve in response to snake venom or did the protease inhibitors evolve to regulate some physiological function controlled by metalloproteinases in resistant animals? In either case, resistant animals would have a survival advantage. Animals that have a natural resistance to metalloproteinases are important sources of proteinase inhibitors that can be used to develop drugs to control metalloproteinase related diseases.

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